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CTCF Regulates Kaposi's Sarcoma-Associated Herpesvirus Latency Transcription by Nucleosome Displacement and RNA Polymerase Programming

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CCCTC-binding factor (CTCF) has been implicated in various aspects of viral and host chromatin organization and transcriptional control. We showed previously that CTCF binds to a cluster of three sites in the first intron of the Kaposi's sarcoma-associated herpesvirus (KSHV) multicistronic latency-associated transcript that encodes latency-associated nuclear antigen (LANA), viral cyclin (vCyclin), vFLIP, viral microRNAs, and kaposin. We show here that these CTCF binding sites regulate mRNA production, RNA polymerase II (RNAPII) programming, and nucleosome organization of the KSHV latency transcript control region. We also show that KSHV bacmids lacking these CTCF binding sites have elevated and altered ratios of spliced latency transcripts. CTCF binding site mutations altered RNAPII and RNAPII-accessory factor interactions with the latency control region. CTCF binding sites were required for the *in vitro* recruitment of RNAPII to the latency control region, suggesting that direct interactions between CTCF and RNAPII contribute to transcription regulation. Histone modifications in the latency control region were also altered by mutations in the CTCF binding sites. Finally, we show that CTCF binding alters the regular phasing of nucleosomes in the latency gene transcript and intron, suggesting that nucleosome positioning can be an underlying biochemical mechanism of CTCF function. We propose that RNAPII interactions and nucleosome displacement serve as a biochemical basis for programming RNAPII in the KSHV transcriptional control region.

Kaposi's sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus 8 (HHV8), is a human gammaherpesvirus that infects ~15% of the world's population and can establish long-term latent infection in B lymphocytes of infected individuals (1–3). The virus was identified originally as the etiological agent associated with all forms of Kaposi's sarcoma (KS) (4) and was subsequently shown to be associated with pleural effusion lymphoma (PEL) and multicentric Castleman's disease (4–6; reviewed in references 2, 3, and 7). Viral pathogenesis and persistence depend on a complex balance between latent and lytic infection. In most latently infected cells, viral gene transcription is limited to a region of the viral chromosome that encodes latency-associated nuclear antigen (LANA/ORF73), viral cyclin (vCyclin/ORF72), vFLIP (ORF71), viral microRNAs (vmiRNAs), and kaposin (K12) (8, 9), which are critical for viral genome maintenance and host cell survival during latent infection (2, 7, 10). The latency transcripts have complex structures, with multicistronic messages and several alternative promoters, as well as lytic gene promoters for both sense and antisense orientations. Proper regulation of these various transcripts is essential for viral persistence and host cell survival.

Transcriptional regulation and mRNA processing of the KSHV major latency transcripts have been investigated in some detail (8, 9, 11–16). Transcription initiation has been mapped to a single major start site that functions largely through a strong core initiator element, with no apparent enhancer or upstream promoter regulatory factors (11, 12). The initiation site of the latency transcript lies within the 5′ untranslated region (UTR) of the complementary strand transcript for K14/ORF74 (viral G protein-coupled receptor [vGPCR]), which is expressed primarily in lytically induced B cells but is also detected in most KSHV-infected tissues and has been implicated in the pathogenesis of KS (17–21). Thus, bidirectional transcription from this region may occur in some pathogenic infections associated with KS. Additionally, an internal initiation site for the LANA transcript can be induced during lytic infection through the activation of Rta binding to a cellular CBF1 site (22). The latency transcript can be alternatively spliced to form the transcript LT1, which expresses LANA, vCyclin, and vFLIP, or LT2, which expresses only vFLIP and possibly vmiRNAs and K12 (9, 14, 16). In addition, an alternative downstream promoter for a transcript that expresses vmiRNAs and K12 has been identified (13). The selection of latency transcripts and promoters is likely to play a significant role in the regulation of KSHV latency, but the details of this regulation remain unknown.

Chromatin-organizing factors and epigenetic modifiers are known to play significant roles in regulating KSHV latency-associated transcription (23–25). We previously showed that the chromatin-organizing CCCTC-binding factor (CTCF) binds to three sites located within the first intron of the major latency transcript (26). CTCF is a multifunctional zinc finger DNA binding protein that can regulate transcription, insulate chromatin, and mediate interactions between spatially separate DNA regulatory elements

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(27). CTCF has also been shown to colocalize with cohesins at multiple sites in the cellular and viral genomes, including sites within the first intron of the latency transcript (28–30). In addition, CTCF can interact with RNA polymerase II (RNAPII) (31), alter nucleosome positions and histone variant enrichment (32), and influence histone modification patterns (33). Previous studies have shown that CTCF binding sites in the KSHV latency control region compromise viral episome maintenance (26) and alter latency transcription control, including deregulation of LANA and derepression of lytic transcripts for K14/vGPCR (34, 35). Other studies revealed that CTCF and cohesin mediate interactions between the latent and lytic control regions (35) and that the loss of cohesin, but not CTCF, leads to derepression of lytic immediate-early gene transcription (34). Whether the DNA-looping function and chromatin-organizing activity of CTCF are related to histone modifications, RNAPII binding, or RNA processing has not been resolved.

Transcription initiation, elongation, and mRNA processing are thought to be coregulated through posttranslation modification of the RNAPII carboxy-terminal domain (CTD) (36). The CTD can be phosphorylated at serine 5 by the initiation factor transcription factor II H (TFIIH) and at serine 2 by positive transcription elongation factor b (pTEFb) (36–39). RNA polymerase elongation and mRNA processing are also regulated by factors that associate with the CTD, including negative elongation factor A (NELF-A) and the positive elongation factor Spt5, both of which function in multiprotein complexes (40). Furthermore, several genome-wide studies revealed that NELF-A colocalizes with RNAPII at chromatin insulators, suggesting that RNA polymerase pausing may be linked to insulator function (41, 42). In addition, mRNA-processing factors, such as polypyrimidine tract binding protein (PTB), and histone modifications, such as H3K36me3, may also be linked to CTD modifications and RNAPII activity (43–46). Thus, regulation of RNAPII may be intimately linked to chromosomal structure and histone modification patterns.

In this work, we used KSHV bacmid mutants to investigate the role of CTCF binding sites within the first intron of KSHV major latency transcript-encoding DNA. We analyzed the genomic stability, transcription profiles, histone modifications, nucleosome organization, and RNAPII processing for latency transcripts of the KSHV genome with wild-type (wt) or mutated CTCF binding sites. We have concluded that CTCF binding sites are important for latency transcription control and that a fundamental aspect of regulation occurs at the level of nucleosome positioning and RNAPII programming mediated by CTCF.

MATERIALS AND METHODS

Cells and bacmids. The KSHV-positive PEL cell line BCBL1 was cultured at 37°C in 5% CO_2 in RPMI medium (Gibco BRL) supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 U/ml). Cells from the 293T cell line (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing penicillin-streptomycin (50 U/ml). The 293T-derived cell lines containing KSHV bacmids were all cultured in a manner identical to that for the 293T cells except for the addition of 200 μg/ml hygromycin B for the selection of recombinant viral genomes. The generation and validation of bacmid mutations at CTCF binding sites were described previously (35). Briefly, Bac36A contains an ampicillin resistance gene inserted between ORF69 and K12 at position 117716 (all KSHV genome positions are based on information from NCBI accession number NC_009333.1). CC-mt1 is a 6-bp substitution mutation in all three CTCF binding sites in the Bac36A background. R-wt1 is the rescue of the CC-mt1 virus by conversion to wt CTCF binding sites in the Bac36A background.

ChIP. Chromatin immunoprecipitation (ChIP) was performed according to the protocol given by Upstate Biotechnology Inc. and as described previously (47). A Diagenode Bioruptor was used to sonicate genomic DNA into 200- and 400-bp DNA fragments according to the manufacturer's protocol. Resultant cell lysates were subjected to immunoprecipitation with the indicated antibodies and decross-linked, and purified DNA was then analyzed using real-time PCR analyses. ChIP values were calculated as fold increases over isotype-specific IgG values for each antibody and primer set. The primer sequences used in this study are available upon request.

Antibodies. The following rabbit polyclonal antibodies were used for the Western blot and ChIP assays: anti-IgG (Santa Cruz Biotechnology), anti-acetylated histone H3 (anti-H3ac) (Millipore), anti-trimethyl histone H3K9, H3K4, H3K36, and H3K79 (Millipore), anti-RNAPII (Abcam), anti-RNAPII S2 (Abcam), anti-RNAPII S5 (Bethyl Laboratories and Abcam), anti-CTCF (Millipore), anti-PTB (Invitrogen), anti-Spt5 (Bethyl Laboratories), anti-proliferating cell nuclear antigen (anti-PCNA) (Abcam), and anti-NELF-A (Bethyl Laboratories).

DNA affinity assay. The DNA affinity assay was conducted as described previously with minor modifications (48, 49). PCR products generated with one 5′-biotin-labeled primer were bound to streptavidin beads. Streptavidin beads (Dynabeads M-280 Streptavidin) (35 μl/sample) were washed two times in 500 μl of 2× bind and wash (B&W) buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 2 M NaCl), washed once with 500 μl of 1× B&W buffer, and linked with PCR products in 100 μl of 1× B&W buffer overnight. Unbound DNA was removed with two 5-min washes with 500 μl of 2× B&W buffer and one 5-min wash with 500 μl of D150 buffer (150 mM KCl, 40 mM HEPES [pH 7.9], 1 mM EDTA [pH 8.0], 2 M NaCl, 0.05% NP-40, 10 mM beta-mercaptoethanol). In a separate tube, nuclear extract was preincubated with sonicated sperm DNA; 125 μl of nuclear extract was preincubated with 350 μl of 0.4 mg/ml salmon sperm DNA at 4°C for 30 min, the mixture was microcentrifuged at 14,000 rpm for 5 min, and then the supernatant of the nuclear precipitate was harvested. Five hundred microliters of supernatant from the nuclear extract was supplemented with 5 μl of 10 mM ATP and reacted for 1 h at 4°C with streptavidin beads that bound PCR products. Streptavidin beads were then washed 6 times (10 min each) with cold D150 buffer at 4°C, with centrifugation at 2,000 rpm for 5 min. The washed beads were resuspended in 25 μl of 2× SDS protein-loading buffer for Western blot analysis. The PCR products used in this DNA affinity assay were produced by amplifying regions 127446 to 128150 from the KSHV Bac36 and KSHV CTCF mutant bacterial artificial chromosome (BAC) (CC-mt1) major latency control regions. An ~700-bp DNA fragment amplified from pUC18 multiple cloning sites was used as an internal control. CTCF, RNAPII, and PCNA were tested by Western blotting to determine whether they bind to the major latency control region of KSHV.

RNA analysis. Total RNA was extracted from KSHV bacmid-transformed 293T cells using an RNeasy kit (Qiagen) and then further treated with DNase I (New England Biolabs). Two micrograms of total RNA was reverse transcribed using random decamers (Ambion) and Superscript II RNase H^- reverse transcriptase (Invitrogen). Semiquantitative and quantitative PCR methods were used. For semiquantitative PCR assays, LTc was assayed with the LTc-F and LT-R primer set. Production of LT1 and LT2 was assessed with the LT1-F and LT-R and the LT2-F and LT-R primer sets, respectively. The LyT-F and LyT-R primer set was used to measure the K14/vGPCR transcript levels. The rLTc-F and rLT-R primer set was used in real-time quantitative PCR (qPCR) assays to measure LTc levels. We used the rLT1-jF and rLT-R and the rLT2-jF and rLT-R primer sets for LT1 and LT2, respectively. The 5′ UTRs of all latent transcripts were measured with primer sets designed from the unspliced 5′ UTR; the primer pair was r5UTR-LT-F and rLT-R. Additionally, we used intron-exon junction-specific primers to quantify the LT1 and LT2 transcripts. The LT1 junction primer (rLT1-jF) spans the KSHV 127462 to 127448

TABLE 1 Primer sequences used for quantification of KSHV latent transcripts

Primer	Positions	Primer sequence	Reference
rLT-R	128027–128046	AATGTGTGTATCATTTGGAG	Reverse
r5UTR-LT-F	127961–127980	CTGTTTATAAGTCAGCCGGA	Forward
rLTc-F	127909–127928	AAGATAAGGGTGCCTTACGG	Forward
rLT1-jF	127462–127448 and 127962–127966	CTCGGGAAATCTGGTTGTTT	Forward (junction)
rLT2-jF	124010–124024 and 127962–127966	CGGCGACGGTGGCTTTGTTT	Forward (junction)
LT-R	127962–127986	GCTTGGTCCGGCTGACTTATAAAC	Reverse
LTc-F	127637–127660	GCATTAAGCTGCAATACCGCCGAT	Forward
LT1-F	126141–126164	GCTCCTGCTGCTGTTGTGAACTTT	Forward
LT2-F	122501–122524	TGACATTAGGGCATCCACGTCAGT	Forward
LyT-R	130243–130259	CGCCTGGCTTGCAGCTT	Reverse
LyT-F	128979–128998	TGGTGGGCCTATTTGGGATA	Forward

and 127962 to 127966 regions. The LT2 junction primer (rLT2-jF) spans the KSHV 124010 to 124024 and 127962 to 127967 regions. Detailed primer sequences are listed in Table 1.

Nucleosome mapping through indirect end labeling. Nucleosome mapping methods were essentially as described previously (29, 50). Briefly, nuclei (10×10^7 nuclei/reaction mixture) from 293T cells carrying KSHV BACs were isolated as described above and digested for 2 min at 37°C with micrococcal nuclease (MNase I) (300, 1,500, or 7,500 U/ml) in MNase I digestion buffer (0.32 M sucrose, 50 mM Tris-Cl [pH 7.5], 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride), followed by the addition of stop buffer and proteinase K for 2 h at 50°C. DNA was extracted with phenol-chloroform, precipitated with ethanol, and subjected to restriction digestion with 500 U of BamHI and 500 U of NheI at 37°C for 16 h. Control genomic DNA from nuclei not treated with MNase I was purified as described above and subjected to identical restriction digestion with BamHI and NheI, followed by treatment with 6 or 60 U/ml MNase I for 5 min at 37°C. DNA was further purified by phenol-chloroform extraction and ethanol precipitation and was analyzed by Southern blotting on 1.7% agarose gels. Southern blots were then probed with digoxigenin (DIG)-labeled PCR products corresponding to the 5′ or 3′ ends of the BamHI or NheI restriction fragments of the KSHV latency control region. PCR DIG probes for indirect end labeling were generated according to the protocol for the Roche PCR DIG probe synthesis kit. The full-length probe (positions 126622 to 128309), left-end probe (positions 126622 to 127006), and right-end probe (positions 127430 to 128309) were generated by PCR and used for Southern blot hybridization.

Mononucleosome enrichment was assessed with qPCR assays with primers spanning every 150 bp across the latency control region. Nuclei from 293T cells carrying KSHV BACs such as R-wt1 or CC-mt1 were treated with MNase I as described above, followed by BamHI-NheI double digestion. DNA fragments of 0.19- to 0.3-kb size were assumed to be on nucleosomes and were analyzed for enrichment of the KSHV latency control region in real-time PCR assays. Quantification of all spots spanning the KSHV latency control region was defined relative to the abundance of the KSHV 126942 to 127006 spot. The primers used to prepare the PCR-amplified DIG probes are available upon request.

Restriction enzyme accessibility assay. Restriction enzyme digestion was conducted with either nuclei isolated from 293T cells carrying KSHV

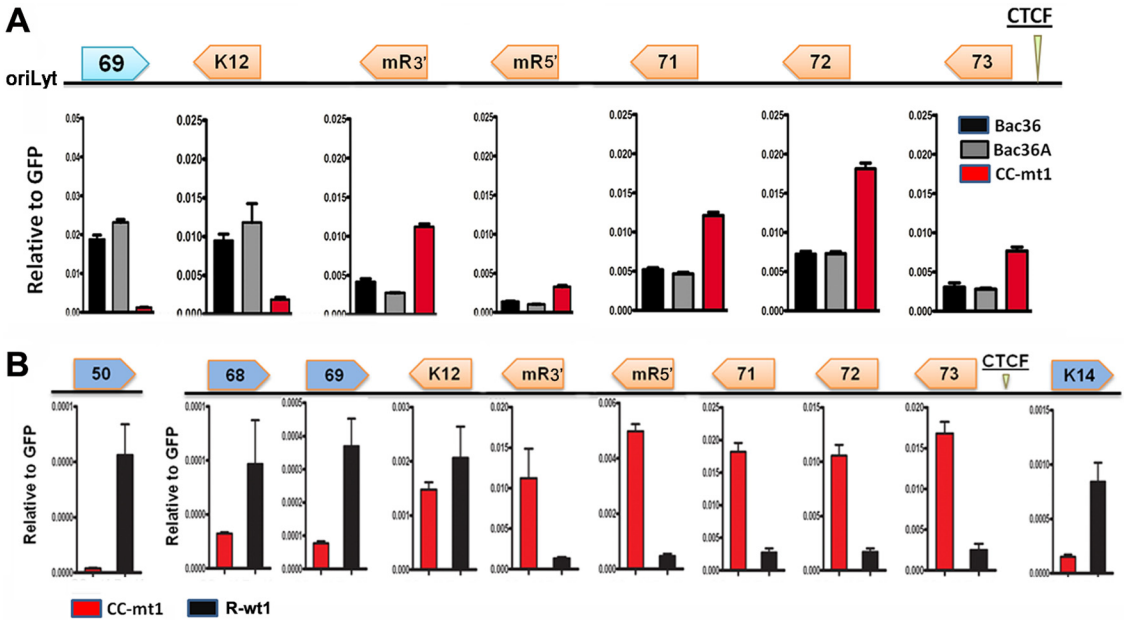

FIG 1 Intragenic CTCF binding sites regulate the KSHV latent and lytic transcript balance. (A) Quantitative RT-PCR analysis of RNA from regions across the KSHV major latency transcription locus in Bac36, Bac36A, or CC-mt1 bacmid genomes in 293T cells. The primers for ORF73, ORF72, ORF71, vmiRNA cluster 5′ (mR5′) (positions 121838 to 121897), vmiRNA cluster 3′ (mR3′) (positions 120526 to 120587), K12, and ORF69 are indicated above each graph. (B) Same as for panel A except that CC-mt1 and R-wt1 bacmid genomes in 293T cells are compared. The primers for K14, ORF73, ORF72, ORF71, mR5′, mR3′, K12, ORF69, ORF68, and ORF50 are indicated above each graph. All viral mRNA values were normalized to bacmid-encoded green fluorescent protein (GFP) mRNA values.

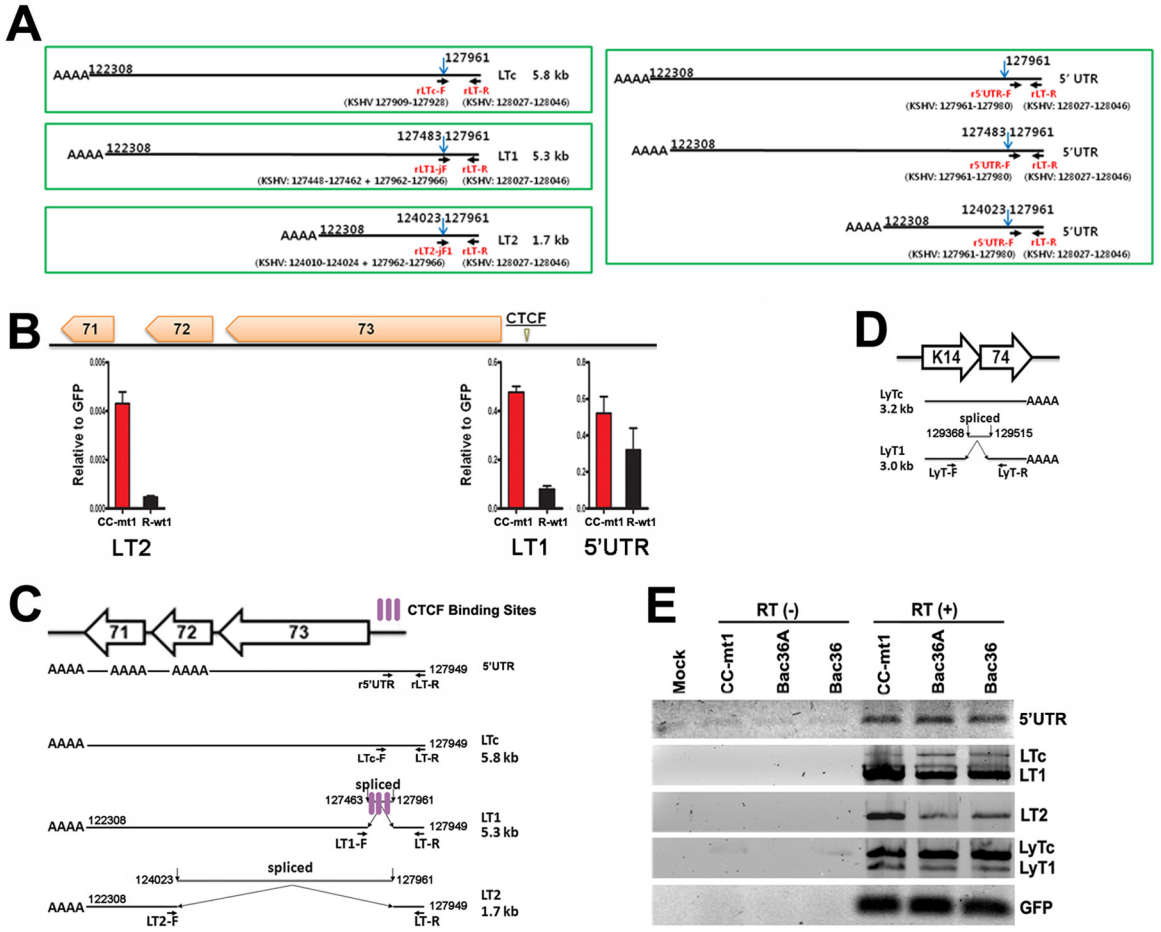

FIG 2 CTCF sites modulate mRNA processing of the KSHV latency cluster. (A) Schematic diagram of previously characterized RNA start and splice sites for the major KSHV latency transcripts, referred to as LTc, LT1, and LT2. Intron-exon junctions and positions of the primers used for the qPCR assays are indicated. (B) Quantitative RT-PCR analysis of 5′-UTR, LT1, and LT2 mRNA transcripts in CC-mt1 or R-wt1 bacmid genomes in transduced 293T cells. Values are relative to those for bacmid-encoded GFP. (C) Schematic diagram of latency transcripts and primer positions for semiquantitative RT-PCR analysis of latency transcripts LTc, LT1, and LT2. CTCF binding sites are indicated in purple. (D) Schematic diagram of lytic transcripts for LyTc and LyT1. (E) Semiquantitative RT-PCR analysis of CC-mt1, Bac36A, and Bac36 bacmids in transduced 293T cells assayed without (-) or with (+) reverse transcriptase (RT). RT-PCR products for the 5′ UTR, LTc, LT1, LT2, LyTc, LyT1, and GFP are indicated.

BACs or purified KSHV BAC DNA only. Nuclei from 10^8 293T cells were purified and incubated with no enzyme, AlwNI (100 U), or BsaI (100 U) for 4 h at 37°C, followed by DNA extraction (total DNA). Control reactions included digestion of purified bacmid DNA from *Escherichia coli*. Extracted nuclear DNA (20 μg) or control bacmid DNA (1 μg) was then digested with BamHI (100 U) and NheI (100 U) for 4 h at 37°C, precipitated with ethanol, and loaded onto 1% agarose gels for Southern blot analysis with PCR DIG probes spanning KSHV genome positions 126622 to 127818.

RESULTS

CTCF sites regulate latency transcription. The CTCF binding site cluster is located in the first intron of the ORF73-ORF72-ORF71 multicistronic transcript. We previously generated substitution mutations in each of the three CTCF binding sites (CC-mt1) and compared that bacmid with the parental wt bacmids (Bac36 and Bac36A) with respect to episomal stability and mRNA expression (35). Consistent with previous studies, we show here that ORF73, ORF72, ORF71, and vmiRNA transcript levels were elevated in the CC-mt1 bacmid lacking CTCF binding sites at the first intron (Fig. 1A). In contrast, K12 and ORF69 mRNA levels were significantly reduced in CC-mt1. This is consistent with previous findings that CTCF binding sites can regulate distal sites through DNA looping, and a DNA loop was observed between CTCF sites and the 3′ end of the K12 transcript (35). To eliminate concerns that the CC-mt1 mutations created an irrelevant gain of function, we generated a CC-mt1 revertant (R-wt1) in which the CTCF binding sites were restored to the wt sequences (Fig. 1B). We found that expression levels of the latency cluster genes ORF73, ORF72, ORF71, and vmiRNA were elevated relative to those of other viral genes. Additionally, we found that lytic gene levels were substantially reduced in the CTCF mutant relative to those in the wt bacmids, although overall expression levels were very low.

Intronic CTCF sites modulate RNA splicing. Since the CTCF binding sites are also located within the first intron, we investigated whether those sites affected mRNA splicing. The major latency transcript can be alternatively spliced to generate two latency transcripts, LT1 and LT2 (Fig. 2A). The CTCF binding sites themselves do not overlap with the splice junction sequences and

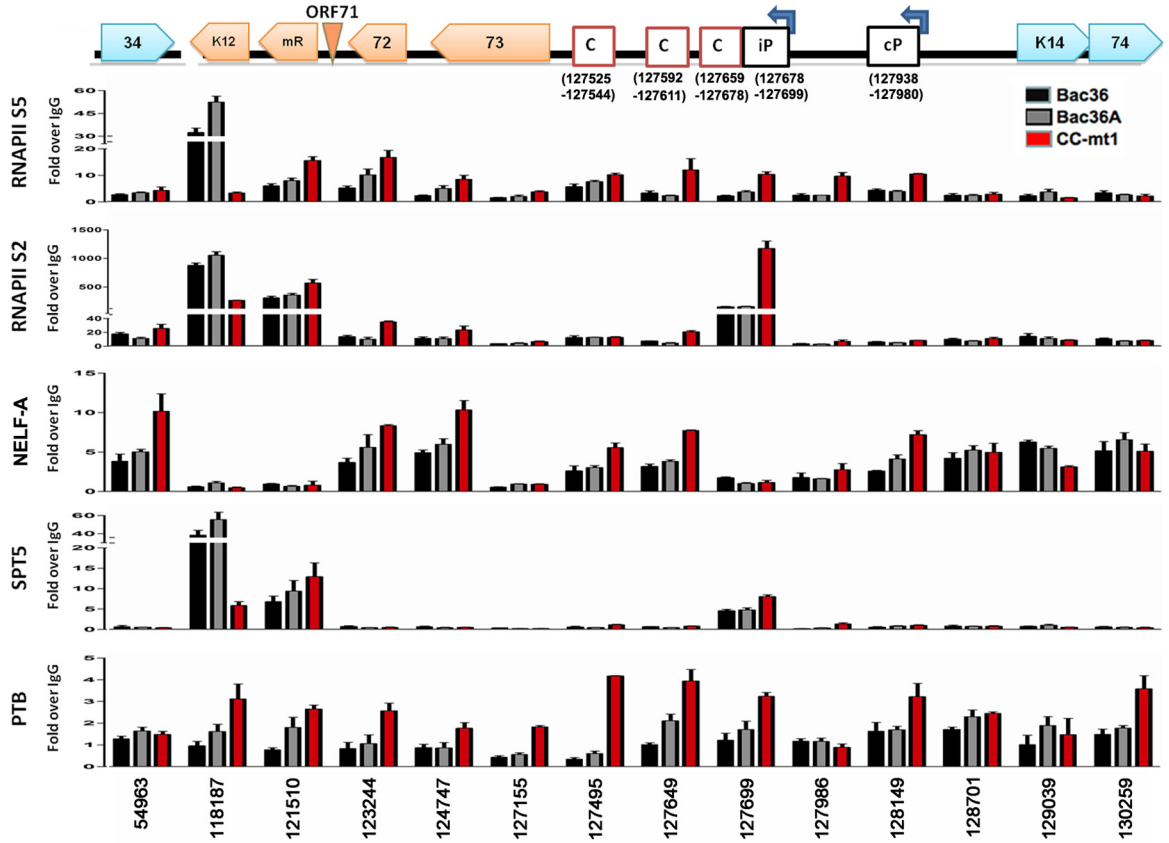

FIG 3 CTCF regulates RNAPII programming in the KSHV latency control region. ChIP assays with anti-RNAPII S5, anti-RNAPII S2, anti-NELF-A, anti-Spt5, or anti-PTB antibodies were performed with CC-mt1, Bac36A, or Bac36 DNA at various sites across the KSHV latency control region, as indicated below each bar graph. Bac36, Bac36A, and CC-mt1 values are shown, with error bars representing the standard deviation from the mean from at least three experimental replicates. CTCF/cohesin binding sites (C), the LANA-inducible promoter (iP), and the constitutive promoter (cP) are indicated.

are not predicted to alter splicing. None of the CTCF mutants is predicted to alter intron splice acceptor or donor recognition. To determine if CTCF binding sites in the first intron affected latency transcription or spliced mRNA products, we investigated the relative levels of formation of the various latency transcripts and their spliced products using exon-intron junction- and size-specific reverse transcriptase (RT) PCR assays (Fig. 2A). Quantitative real-time PCR analysis using intron-exon junction-specific primers revealed that CTCF mutation resulted in substantial increases in spliced transcript levels, with only marginal increases in parental transcript (e.g., 5′-UTR) levels (Fig. 1B). Using conventional PCR assays in which specific PCR product sizes were validated by gel electrophoresis, we found that CTCF mutants generated ~2-fold-higher levels of the LT1 and LT2 splice products than the parent 5′-UTR or unspliced LTc transcript. No significant changes in the levels of K14 or the ORF74 transcript products LyTc or LyT1 were detected. These findings suggest that CTCF binding in the first intron of the latency gene transcript can modulate mRNA splicing.

CTCF sites alter RNA polymerase modification and elongation factor assembly. One potential mechanism through which intragenic CTCF binding may affect transcription would be to alter RNAPII or RNAPII-associated factors that affect transcription elongation or mRNA processing. We therefore assayed the relative enrichment of RNAPII (phospho-isoforms S5 and S2), NELF-A, Spt5, and PTB across the latency transcript of KSHV bacmid genomes with wt or mutant CTCF binding sites (Fig. 3). In wt bacmid genomes, we found that RNAPII S2 was enriched at the LANA-inducible promoter (iP) region (position 127699), while RNAPII S2, RNAPII S5, and Spt5 were enriched at the 3′ end of the latency transcription cluster near the K12 transcription start site (KSHV position 118187). NELF-A and PTB were not enriched at any specific position in the latency control region. In the CTCF mutated bacmid (CC-mt1), we observed an ~3- to 4-fold increase in RNAPII S5 levels throughout the first 300 bp of the latency transcript, while RNAP S2 levels increased ~2-fold at the LANA iP and CTCF binding sites. Interestingly, both RNAPII S5 and RNAPII S2 showed reduced binding at the K12 gene, which corresponds to the loss of K12 transcription observed for the CC-mt1 genome (Fig. 1). PTB and NELF-A binding increased at several positions across the latency transcripts, including the mutated CTCF binding sites, in CC-mt1, relative to that in wt genomes. These findings suggest that intragenic binding of CTCF regulates RNAPII and RNAPII-associated factor interactions at positions proximal and distal to the CTCF binding sites.

Intragenic CTCF can interact with RNAPII *in vitro*. CTCF has been shown in coimmunoprecipitation assays to interact weakly with RNAPII. We wished to test whether CTCF could influence the binding of RNAPII to the KSHV latency locus when bound to intronic DNA. We therefore tested the ability of CTCF wt or mutant DNA encompassing the complete first intron and

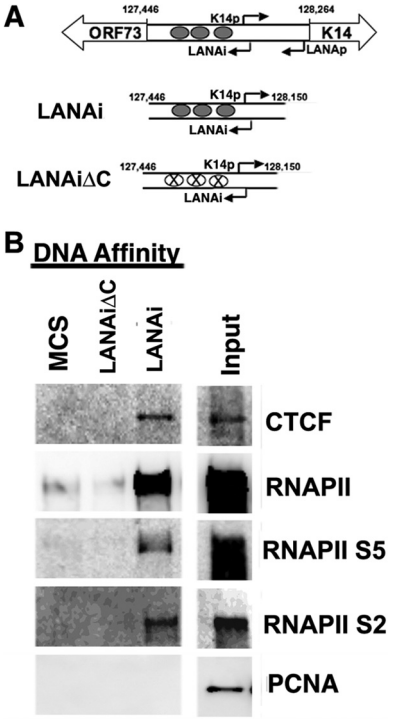

FIG 4 CTCF stabilizes RNAPII binding to the latency control region. (A) Schematic diagram of DNA fragments (LANAi and LANAiΔC) used for DNA affinity assays. Wild-type (filled ovals) and mutant (crossed ovals) CTCF binding sites located within the first intron and the relative locations of the K14 or lytic promoter (K14p), the inducible LANA promoter (LANAi)), and the latency-associated LANA promoter (LANAp) are indicated. (B) DNA affinity assay with 293T cell nuclear extracts incubated with control DNA (multiple cloning sites [MCS]), LANAiΔC, or LANAi and then assayed by Western blotting with antibodies to CTCF, RNAPII, RNAPII S5, RNAPII S2, or PCNA, as indicated. Inputs (10% of total 293T cell nuclear extracts) are shown.

inducible LANA (LANAi) promoter region (KSHV coordinates 127446 to 128150) to recruit RNAPII from nuclear extracts (Fig. 4). We found that wt DNA (LANAi) but not a CTCF mutant (LANAiΔC) efficiently bound to both CTCF and RNAPII. We also observed binding for both the S5 and S2 isoforms of RNAPII, although the S2 form bound to a greater extent. Neither form of RNAPII bound to the CTCF mutant probe (LANAiΔC) or control DNA. PCNA did not bind to any of the probes, indicating that the interactions of CTCF and RNAPII with LANAi were relatively specific. These findings indicate that CTCF binding to the first intron of the latency transcript control region can interact with RNAPII and can interact selectively with the S2 isoform of RNAPII associated with transcription elongation.

CTCF binding limits enrichment of histone H3K4 methylation and acetylation within the major latency transcript. Histone modifications have been linked to various RNAPII functions, including initiation, pausing, and splicing. To determine if CTCF binding altered the histone modification pattern in the latency transcription region, we assayed for the relative enrichment of histones H3K4me3, H3acetyl (H3ac), H3K79me3, H3K36me3, and H3K9me3 (Fig. 5). Several significant differences were observed between CC-mt1 and the control wt genomes of Bac36 or Bac36A. We found a significant increase in H3K4me3 levels at mutated CTCF binding sites. However, we also observed relative increases in all histone modifications at this region, suggesting that nucleosome occupancy may also increase with histone H3K4me3 modification. In contrast, there were corresponding reductions in most histone modifications at the K14-ORF74 locus. At the 3′ end of the latency control region, near the K12 promoter (position 118187), we observed reductions in H3ac levels and increases in H3K36me3 and H3K9me3 levels in CC-mt1 relative to those in the wt genomes. These histone modifications at the K12 promoter correlate with the reduction in K12 transcription observed in CC-mt1 relative to that observed in wt DNA (Fig. 1). Together, these findings suggest that CTCF binding alters histone modification and nucleosome density at several positions in the latency control region.

CTCF binding disrupts ordered nucleosome phasing within the latency transcription unit. CTCF binding may influence nucleosome organization, which in turn could influence histone modifications and RNAPII function and interactions. To investigate the role of CTCF sites in nucleosome organization at the KSHV latency transcription locus, we performed MNase I digestion followed by indirect end labeling of either R-wt1 (with CTCF sites intact) or CC-mt1 (with three CTCF sites mutated) KSHV bacmid genomes maintained in 293T cells (Fig. 6A to D). For indirect end-labeling studies, nuclei were digested with MNase I, extracted from nuclei, and then digested with NheI and BamHI to liberate a fragment from position 126622 to position 128308 spanning the CTCF binding sites. The digestion patterns of total cellular DNA from CC-mt1- and R-wt1-transformed 293T cells were indistinguishable when examined with ethidium bromide staining of agarose gels (Fig. 6A). Southern blots of digested DNA revealed significant differences in MNase I digestion patterns within the KSHV latency control regions of the CC-mt1 and R-wt1 bacmid genomes (Fig. 6B to D). A probe spanning the entire region (positions 126747 to 127989) revealed that a major MNase I cleavage site is present in the R-wt1 bacmid genome but is absent in the CC-mt1 bacmid genome. This MNase I site appears to overlap the CTCF sites and may reflect the stable binding and MNase I resistance of the three CTCF binding sites. In the CC-mt1 genome, in which the CTCF binding sites are mutated, the MNase I pattern is regularly phased, with nucleosomes evenly spaced every ~150 bp. Indirect end labeling with probes specific for each end of the NheI-BamHI restriction fragment (Fig. 6C and D) suggested that a single nucleosome is weakly positioned between the transcription initiation site and the CTCF sites and that a series of 2 or 3 more strongly positioned nucleosomes are located downstream of the CTCF sites in the R-wt1 bacmid. In contrast, nucleosome positioning is more regularly phased at both ends in the CC-mt1 bacmid genome. To further investigate the nucleosome occupancy of the latency transcript region, we quantified the DNA associated with the mononucleosome fraction of MNase I digests from wt and mutant bacmids (Fig. 6E). Mononucleosome DNA was quantified with a series of primer pairs that span ~50-bp regions across the latency transcript (Fig. 6E). Consistent with the indirect end-labeling results, we found that the wt bacmid had relatively less DNA associated with the mononucleosome fraction, while the CC-mt1 bacmid had much greater enrichment and a pattern consistent with four nucleosomes spanning the region immediately downstream of the transcription initiation site (summarized in Fig. 6F).

CTCF binding increases DNA accessibility near the splice acceptor site. To further investigate the chromatin structure of the

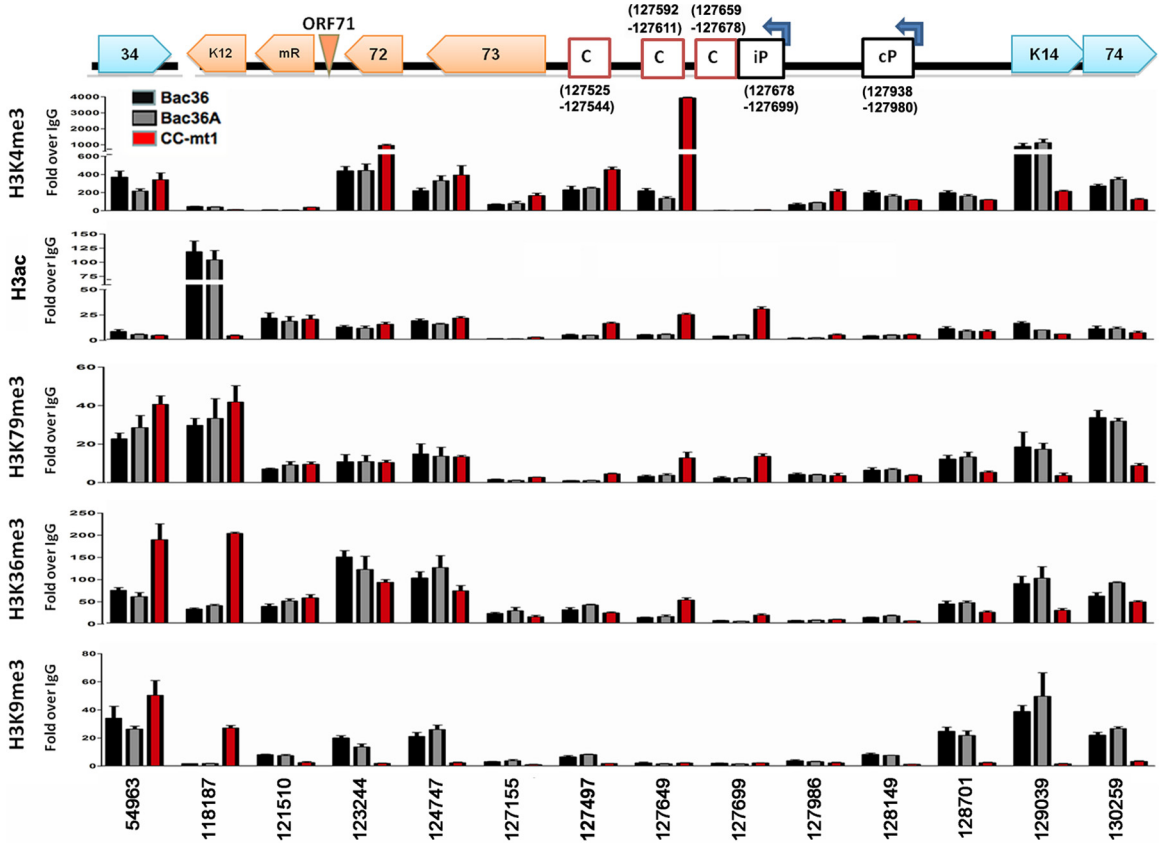

FIG 5 CTCF binding affects histone modification patterns throughout the major latency transcript. Bac36, Bac36A, and CC-mt1 genomes were assayed by ChIP for H3K4me3, H3ac, H3K79me3, H3K36me6, and H3K9me3 at the indicated positions across the KSHV latency control regions. C indicates CTCF/cohesin binding sites, and iP and cP indicate the inducible promoter and constitutive promoters of KSHV major latent transcript, respectively.

region surrounding the CTCF binding sites in the KSHV latency transcript, we utilized a restriction enzyme accessibility assay. Restriction enzyme accessibility is assayed with freshly isolated nuclei that retain most chromatin structures formed in living cells. We compared the restriction enzyme accessibility of AlwNI and BsaI on nuclei isolated from CC-mt1 or R-wt1 bacmid genomes in 293T cells. AlwNI cleaves at position 127618, between CTCF binding sites 1 and 2, while BsaI cleaves at position 127353, which is downstream of CTCF site 3 and just downstream of the 3′ splice acceptor site for the first intron of LT1. We found that BsaI cut the R-wt1 but not the CC-mt1 DNA, while AlwNI did not cut either R-wt1 or CC-mt1 DNA (Fig. 7A). AlwNI and BsaI cut purified bacmid DNA from R-wt1 and CC-mt1 with similar efficiencies (Fig. 7B), indicating that nucleosome-free DNA is completely accessible to these enzymes. The increased accessibility of R-wt1 DNA to BsaI suggests that this region is not protected by a nucleosome and that an intact CTCF binding site is required to maintain the accessibility of this site. These findings are consistent with the MNase I nuclease protection assay results, which indicate that CTCF disrupts nucleosome occupancy in the region surrounding the first intron (Fig. 7).

DISCUSSION

CTCF can function as a central regulator of chromatin structure and gene expression, but the underlying biochemical mechanisms of these various functional outcomes remain enigmatic. Here, we have explored the functional and biochemical properties of CTCF binding to a cluster of three sites in the first intron of the KSHV major latency transcript. We show that mutation of these sites leads to significant changes in viral gene expression, mRNA processing, RNAPII enrichment, RNAPII-accessory factor interactions, histone modifications, and nucleosome positioning. CTCF can bind and bend DNA (51), interact with the RNAPII large subunit (52), and bind cohesin subunits (53) and other chromosomal regulatory factors (54) that may mediate some of these complex functions. How each of these biochemical activities contributes to the functional properties of CTCF binding remains to be determined. In this study, we provide evidence that RNAPII interactions and nucleosome disruption play important roles in how CTCF regulates transcription from the KSHV latency control region.

CTCF changes RNA polymerase II programming. Several studies have shown that RNAPII can be programmed to modulate transcription initiation, elongation, and mRNA processing (36, 38). CTCF has been implicated in programming RNAPII pausing at the mammalian tumor necrosis factor alpha (TNF-α) gene (55) and at *Hox* gene insulators in *Drosophila* (41, 56). CTCF was also found to localize with RNAPII elongation factors, including NELF, at the U2 and β-actin genes (57). In this study, we tested the role of CTCF binding sites in regulating RNAPII programming and RNAPII-accessory factor accumulation in the latency transcript control region. Mutation of CTCF binding sites in the first

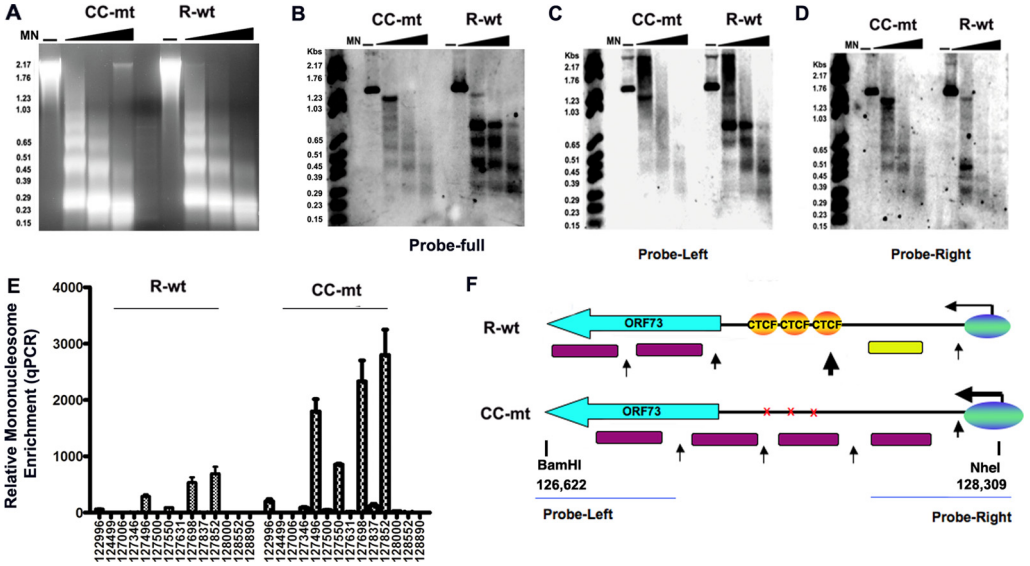

FIG 6 CTCF disrupts nucleosome phasing in the major latency control region. (A to D) MNase I nuclease mapping of nucleosome positions across the latency control regions for CC-mt1 and R-wt1, using indirect end-labeling methods. DNA from MNase I-digested nuclei was extracted and then cleaved with BamHI and NheI prior to gel electrophoresis. (A) Ethidium bromide staining of total cellular DNA after MNase I treatment. (B) Southern blot analysis of MNase I-digested DNA with a probe encompassing the BamHI-NheI fragment (positions 126622 to 128308). (C) Same Southern blot stripped and hybridized with the left-end probe (KSHV positions 126622 to 127006). (D) Same Southern blot stripped and hybridized with the right-end probe (KSHV positions 127430 to 127837). (E) Mononucleosome enrichment assayed by qPCR analysis with primers spanning every 150 bp across the latency control region. The abundances of all fragments spanning the KSHV latency control region were relative to the abundance of the KSHV 126942 to 127006 fragment. (F) Schematic summary of nucleosome positions across the CC-mt1 and R-wt1 latency control regions. The yellow rectangle represents a poorly positioned nucleosome, while the purple rectangles are uniformly phased nucleosomes.

intron of the latency transcript resulted in increases in total latency transcript levels relative to those in other regions of the genome (Fig. 1). This corresponded to increased expression of the spliced (LT1 and LT2) mRNAs relative to total (5′ UTR) mRNA expression (Fig. 2). These findings are consistent with a previous study that showed that LANA mRNA and protein levels were elevated in CTCF site-mutated genomes (35). Our new findings suggest that CTCF binding in the intron limits RNAPII transcription and RNA splicing across this first intron. Mutations of the CTCF binding sites also resulted in increased accumulations of RNAPII and the elongation factor Spt5 at the intronic region (Fig. 3). These results are partly consistent with those of a previous study that showed that RNAPII accumulated with the elongation factor Spt5 at the 5′ end of the first intron of the latency transcripts in latently infected BCBL1 cells (58). Cell type differences and the use of bacmid genomes in the present study may partly explain some of the discrepancies with the earlier studies. While CTCF does not appear to function as a physical block to RNAPII elongation, it may regulate programmed pausing and splicing at intron-exon junctions. CTCF binding sites were sufficient to cause the recruitment of RNAPII to intron-containing DNA *in vitro* (Fig. 4). This recruitment selectively enriched for the S2 phospho-isoform, which is competent for elongation. CTCF binding sites were also important for limiting the accumulation of PTB at the intronic region (Fig. 5). This is consistent with the role of PTB in regulating mRNA splicing and further implicates CTCF binding sites in mRNA processing of the latency transcripts.

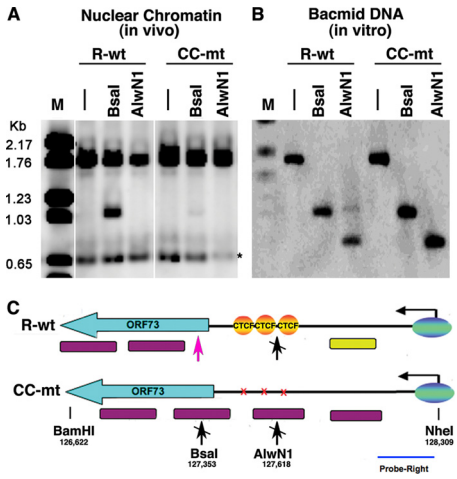

FIG 7 CTCF binding increases restriction enzyme accessibility within the latency control region. (A) Nuclei from 293T cells containing R-wt1 or CC-mt1 bacmid genomes were incubated with either no enzyme (−), BsaI, or AlwNI. DNA was then purified, cut with BamHI and NheI, and then subjected to Southern blot analysis with a probe spanning positions 127430 to 127837. The asterisk indicates a nonspecific partial-digest product. M, molecular mass markers. (B) Purified R-wt1 or CC-mt1 bacmid DNA was cleaved with either no enzyme (−), BsaI, or AlwNI, digested with BamHI and NheI, and then assayed by Southern blotting as described for panel A. (C) Schematic summary of restriction enzyme results. The pink arrow indicates increased BsaI sensitivity in R-wt1 relative to that in CC-mt1 genomes.

Distal effects of CTCF mutations. Mutations in CTCF binding sites at the LANA intron also affected RNAPII and histone modifications at the K12 transcription start site located ~10 kb downstream of the CTCF sites. We found that disruption of CTCF binding resulted in losses of RNAPII S2, RNAPII S5, and Spt5 binding at the K12 transcription start site (Fig. 3). We also observed a loss of H3ac and increases in H3K36me3 and H3K9me3 levels (Fig. 5). These findings are consistent with reductions in

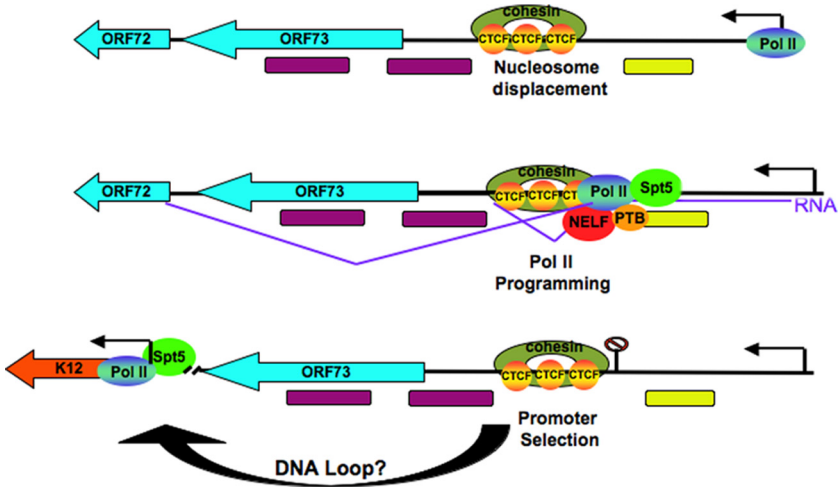

FIG 8 Schematic model of RNA polymerase regulation through CTCF disruption of intragenic histone positioning. Histone positions are indicated by the purple rectangles. Yellow rectangle indicates first nucleosome downstream of the transcription start site and enriched in H3K4me3. RNA polymerase positions and pausing are indicated at splice junctions and the CTCF-cohesin boundary. The purple lines represent mRNA and alternative splice products. CTCF-cohesin was shown to disrupt nucleosome positioning downstream of RNA polymerase II (Pol II) initiation, to alter RNA Pol II-associated factors, and to control transcription initiation site selection.

K12 transcription levels (Fig. 1). In a previous study, it was found that CTCF sites in the LANA intron mediate a stable DNA loop with the 3′ end of the latency transcript region (35). CTCF has a well-established role in mediating long-distance DNA interactions, which have been implicated in promoter-enhancer regulation. Our new findings suggest that CTCF effects on RNAPII function and nucleosome positioning may be coordinated with DNA loop formation between the LANA intron and the 3′ end of the ~10-kb latency transcription unit.

CTCF influences nucleosome position and histone modification patterns. Examination of the histone modification pattern in KSHV bacmids lacking CTCF binding sites revealed several significant changes. The most significant observation was that CTCF binding prevented excessive enrichment of H3K4me3 at the LANA-inducible promoter region (Fig. 3). H3K4me3 is known to form at the first or second nucleosome positioned downstream of the transcription start site (59). Our findings suggest that CTCF prevents the enrichment of H3K4me3 at this position. Since the CTCF position is also located immediately downstream of the inducible lytic promoter for LANA, it is possible that CTCF prevents the aberrant utilization of this alternative internal initiation site during latency. Preventing the enrichment of histone H3K4me3 at the first intron could potentially restrict internal promoter utilization. However, it remains unknown whether CTCF prevents histone H3K4me3 formation directly or interferes with RNAPII elongation and subsequent recruitment of RNAPII-associated histone methyltransferases.

One potential mechanism through which CTCF prevents histone H3K4me3 enrichment is by preventing nucleosome phasing immediately downstream of the transcription start site. Nucleosome positioning has been implicated in the regulation of RNAPII initiation and elongation (40). CTCF has been implicated in nucleosome organization through its ability to position an array of nucleosomes at transcription start sites, as well as localizing with histone variant H2A.Z (32). We were unable to observe significant enrichment of H2A.Z at the CTCF sites in the KSHV latency cluster (data not shown). However, CTCF binding clearly disrupted the regularly spaced phasing of the first few intragenic nucleosomes, as monitored by indirect end labeling of MNase I partial digests (Fig. 6) or qPCR analysis of mononucleosomes (Fig. 6E). Furthermore, CTCF altered restriction enzyme accessibility at a position just downstream of the CTCF binding sites, suggesting that nucleosome phasing is altered relative to genomes lacking CTCF binding sites (Fig. 7). While it is not yet known whether the three CTCF sites can occupy the same DNA that is bound to a nucleosome, our data suggest that CTCF displaces a nucleosome from this chromatin region. The displaced nucleosome could alter histone modification patterns and RNAPII programming, which could have significant effects on RNAPII elongation, internal initiation, and mRNA processing. We therefore propose that the intragenic cluster of CTCF sites displaces a nucleosome and alters the normal phasing typically induced by RNAPII at transcription start sites (Fig. 8).

Chromatin structure, including nucleosome positions and histone modifications, has been implicated in the control of RNAPII initiation, elongation, and mRNA processing (38, 43, 45, 46, 60). Our data suggest that nucleosome displacement may be a fundamental mechanism through which CTCF confers many of its more-complex activities. Nucleosome displacement could account for changes in histone modifications, especially since many modifications are processive and require phased nucleosomes for signal propagation. Nucleosome displacement could also account for the modulation of RNAPII activity and programming, perhaps by reinforcing CTCF as a physical barrier to RNAPII elongation. Nucleosome displacement could also facilitate CTCF functions in DNA looping, potentially by trapping cohesins in nucleosome-free regions. Whether nucleosome displacement is a universal property of all CTCF binding sites or is a common feature of other DNA binding proteins remains to be determined. Nevertheless, our findings strongly support a role for nucleosome displacement as a core biochemical feature of CTCF in the control of KSHV latency transcription.

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